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<p>In Year 1 of this grant, we are establishing procedures for determining the ability of the phosphonic acid diphenylureas to inhibit PC3 prostate carcinoma cells in the nude mouse xenograft. The initial experiments have not confirmed that the phosphonic acid diphenylureas inhibit prostate cancer in the nude mouse xenograft. Additional experiments are necessary to determine the reason for the differences in results in the in vitro and in vivo experiments. We postulate that the i.p. injection of the treatments into the peritoneal cavity does not deliver the phosphonic acid diphenylureas to the tumor.</p> <p>In other experiments, the phosphonic acid diphenylureas have been shown to be potent inhibitors of metalloproteinase (MMP) activity in prostate carcinoma cells. We postulate that these compounds inhibit MMP secretion or synthesis. Additional experiments are proposed to test this hypothesis.</p>				
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FOREWORD

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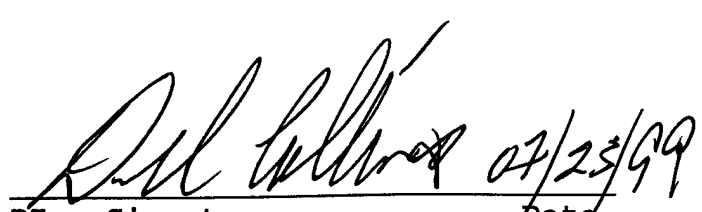
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(5) INTRODUCTION:

We have synthesized and characterized a unique group of diphenylureas that are much more potent than suramin or its sulfonated analogues as inhibitors of cell growth, migration and matrix metalloproteinase activity (MMP-2 and MMP-9) in human microvascular endothelial and the human prostate cancer cell lines (PC3, LNCaP.FGC & DU145), in vitro. Toxicity studies in mice indicate no significant toxicity by the diphenylureas and a half-life 10-fold less than suramin. However, the diphenylureas do not bind the heparin-like growth factors. These results suggest that the diphenylureas may be potent therapeutic agents that will significantly reduce morbidity in man.

The experiments described in this proposal will be the first in vivo test of the ability of a unique group of diphenylureas to inhibit the growth and metastasis in human androgen dependent (LNCaP) and androgen independent (PC3) prostate tumor xenografts in nude mice. We propose that the diphenylureas are potent inhibitors of tumorigenesis, angiogenesis and metastasis in human prostate cancer growth. Furthermore, the antineoplastic activity of the diphenylureas is not related to the inhibition of the binding of the growth factors as has been demonstrated for suramin. These hypotheses will be tested by the following questions:

Question #1. Are selected diphenylureas that inhibited growth of human prostate cancer cell lines in vitro able to inhibit the growth of human androgen dependent (LNCaP) and androgen independent prostate cancer tumors in the athymic nude mouse model?

Question #2. Do the selected diphenylureas inhibit tumor angiogenesis in prostate cancers in vivo?

Question #3. Do the selected diphenylureas inhibit the metastatic ability of prostate cancers in vivo?

Positive results from these experiments will lead to translational research and clinical testing of these diphenylureas for prostate cancer therapy in man during Phase 2 of this proposal. .

(6) BODY:

The work on this grant has focused primarily on Task 1. Determine the effect of the three selected diphenylureas (NF681, NF162 and NF050) and suramin at three dose levels on the growth of the androgen independent human prostate cancer (PC3) in the athymic nude mouse xenograft. This animal protocol requires 10 animals per group and 160 animals for this experiment (months 1-12).

- Carry out the experimental protocol to test the ability of the selected diphenylureas to inhibit the androgen independent human prostate cancer (PC3) in the athymic nude mouse xenograft model (month 1-5.)
- Carry out the bromodeoxyuridine (BrdU) incorporation experiments to determine the growth rate of the prostate tumor under the various treatment conditions (month 5-8).

- Determine the effect of the three selected diphenylureas and suramin on the rate of apoptosis using the Apotag Apoptosis kit (month 5-8).
- Determine the effect of the three selected diphenylureas and suramin on the presence of the matrix metalloproteinases, MMP-2 and MMP-9, by immunohistochemistry (month 8-12).
- Determine the effect of the three selected diphenylureas and suramin on the presence of inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-2, by immunohistochemistry (month 8-12).

Experiment #1: Establish Growth Characteristics of PC3 Cells in Nude Mice in vivo.

Twelve nude mice were injected subcutaneously (s.c.) in each flank with media containing PC3 cells obtained from ATCC. Beginning five days later, six mice were injected intraperitoneally (i.p.) with saline containing 140 μ moles of NF681/kg body weight on alternate days for a total of five days. The remaining six mice were injected with saline only on the same regimen. Animals were visually observed for the next 90 days for visible tumors. Both of the injection sites were checked and no tumors were found. The animals were euthanized and the injection sites were carefully inspected for abnormal areas that might have been minute tumors. Only one animal showed a suspicious area. H & E-stained sections of the excised tissue were examined by the clinical pathologist and no tumor was found.

Results: No tumors were formed in any of the 12 mice injected with PC3 cells obtained from ATCC after 92 days of observation. These results were unexpected and led us to consult with other groups who have used this nude mouse model.

Experiment #2: Effect of Matrigel on Tumor Growth in Nude Mice in vivo.

Nine mice were injected with PC3 cells suspended in Matrigel at three sites on each animal (nape of neck and each flank). After nine days, tumors had formed at each injection site in eight of the nine animals while the remaining animal had tumors on two of three sites. The animals were then separated into control and treatment groups so that each group contained comparable numbers/size of tumors. Animals were injected i.p. with either 140 μ moles/kg body weight saline containing NF681 or saline only for six consecutive days before alternating days of injection for the remaining 22 days of the treatment period (11 additional injections). Animals were sacrificed on day 29 and tumors were taken. Tumor weights and volumes for this experiment are shown in Table 1.

Results: Nude mice injected with PC3 cells suspended in Matrigel showed tumors more rapidly than animals injected with media containing PC3 cells in Experiment #1. Tumors in the Control 2 animal appeared to spontaneously regress during the last six days of the treatment period. The results from this animal were excluded from the control mean. The mean weight of the tumors (gm) and the tumor volume (mm^3) were not significantly different in the control animals compared to those animals treated with 140 μ moles/kg body weight after 22 days of treatment.

Table 1. Tumor weights and volumes for nude mice injected s.c. with PC3 prostate cancer cells in Matrigel™. Treatment animals were injected i.p. with 144 μ moles/kg mouse body weight of NF681 in 0.4 ml saline daily for 6 days and ever second day thereafter for 22 days. Control animals received equal amounts of saline only on the same treatment schedule. Tumors were measured, excised and weighed on day 29 of the experiment.

Animal	Tumor Weight in grams	Tumor Volume in cubic mm
NF681-1	2.0833	680.0
NF681-2	2.0512	2048.0
NF681-3	1.1663	1023.5
NF681-4	1.3798	1966.0
Mean	1.6702	1429.375
Sd	0.467	682.388
Control 1	0.9239	555.3
Control 2	no data	no data
Control 3	3.068	1598.0
Control 4	1.4625	906.0
Mean	1.8181	1019.767
Sd	1.115	530.578

Experiment #3: Growth Characteristics of PC3 Cells in Nude Mice in vivo.

Eighteen athymic nude mice were injected s.c. in each flank with media containing PC3 cells. Beginning ten days after the injections, the animals were divided randomly into three treatment groups: control, NF681 and NF050. Each animal was treated daily with 140 μ moles/kg body weight for ten consecutive days. Subsequently, the injection regimen was changed to daily injections for five days, followed by no injections for two days for the remaining 28 days of the experiment. Tumor weights and volumes are shown in Table 2.

Results: Tumor weights and tumor volumes do not show a reduction in PC3 tumors in animals treated with 140 μ moles/kg body weight of NF681 or NF050. It was noted during autopsy that a white precipitate was deposited in the peritoneal cavity at the injection site. Some of this white material was collected and subjected to HPLC chromatography as described by Kassack and Nickel (1996). The white material appeared to be NF681 or NF050, based on the retention time on the HPLC column.

We are continuing experiments on this problem. It appears that NF681 and NF050 precipitate after injection into the peritoneal cavity. This may be expected since the pH of NF681 and NF050 in water is 8.5 and 9.0, respectively. Thus, they may precipitate out in the slightly acidic pH of the peritoneal cavity and not reach the tumor site. We are working with Dr. Nickel to develop analogues of NF681 and NF050 that have lower pH and will be more soluble in blood and tissue. We also propose to test the effects of NF681 and NF050 in the nude mouse in vivo protocol by injecting the compounds at the site of the tumor. This procedure has been used by other investigators to test the anti-tumorigenic effect of compounds.

Experiment #4: Development of a Method for Quantitation of Microvessel Density in Prostate Tumor Tissue.

Tissues for microvessel density counting have been processed, embedded in paraffin and sectioned onto slides but no immunohistochemical results for the cell surface markers of endothelial cells (Factor VIII, CD31, or CD34) are available at this time. We expected the clinical laboratory to perform either the Factor VIII or CD31 immunohistochemical stains. However, neither of these procedures worked on our tissues since the antibody used for the immunohistochemical staining of Factor VIII was a monoclonal antibody specific for human Factor VIII and had no cross reactivity for murine Factor VIII-related antigen. The same was true for the CD31 and CD34 stains. It is necessary to stain for murine Factor VIII-related antigen, murine CD31 or CD34 since the microvessels that develop in response to the human xenograft are of murine origin. Our laboratory has been able to stain for Factor VIII in these tissues using a polyclonal biotinylated rabbit anti-human Factor VIII (DAKO Code # A0082, DAKO Corporation, Carpinteria, CA) that has a 30% cross-reactivity with murine Factor VIII (technical service representative at DAKO,).

Table 2. Tumor weights and tumor volumes for athymic nude mice injected s.c. with PC3 prostate carcinoma cells in culture medium. Beginning 10 days after the cells were injected, control animals were injected i.p. with 0.4 ml of vehicle solution for 10 consecutive days. Thereafter, 5 days of treatment were followed by 2 days of no treatment for an additional 4 weeks. Treatment animals were injected with 140 μ moles/kg of mouse body weight of either NF681 or NF050 in 0.4 ml of vehicle solution on the same treatment schedule. Tumors were measured, excised and weighed on day 48 of the experiment.

Animal	Tumor Weight in grams	Tumor Weight in cubic centimeters
Control 1	0.603	688.5
Control 2	1.057	382.5
Control 3	no data	
Control 4	0.827	2162.5
Control 5	2.751	1872.0
Control 6	no data	
mean	1.789	2017.250
sd	0.979	872.658
NF681-1	0.679	1303.0
NF681-2	0.583	1694.0
NF681-3	0.659	804.5
NF681-4	0.448	78.0
NF681-5	1.400	1827.0
NF681-6	no data	
mean	0.924	952.500
sd	0.372	715.166
NF050-1	0.720	1300.0
NF050-2	1.240	1586.0
NF050-3	0.522	332.5
NF050-4	0.925	390.5
NF050-5	0.521	224.0
NF050-6	no data	
mean	0.723	307.250
sd	0.304	628.538

Two methods of target retrieval (heated citrate buffer and trypsin digestion) were used in different slides from the same tissues to expose the cell surface antigen from the protein-bridges created in formalin-fixed tissues. The trypsin digestion resulted in better target retrieval but, at present, our procedure results in background staining too high to use an image analyzer to quantitate the results. We have attempted to reduce this background stain by using blocking agents against biotin, avidin and protein. While this has helped, there is still too much background staining, possibly due to the human immunoglobulins present in the human xenograft reacting with the rabbit anti-human immunoglobulin at the concentration necessary to stain the murine Factor VIII. We are attempting to eliminate this problem with background stain by using a biotinyl tyramide reaction. This procedure has been reported to result in much more intense staining with the use of less primary and secondary antibodies (Hunyady et al., 1996).

Martin et al. (1997) reported a study using the three most commonly used monoclonal antibodies (Factor VIII, CD31 and CD34) to assess angiogenesis in human breast cancer tissues. Their findings showed that Factor VIII antibody stained mainly the larger microvessels well (99% of the section on the first run) but also stained other cells (tumor, inflammatory cells and connective tissue), making it difficult to quantitate the microvessels. The CD34 stain was also effective (98% on the first run) in staining endothelial cells but was more specific, staining single isolated endothelial cells as well as the larger microvessels. CD31 was less effective (87% with amplification on the first run), staining single endothelial cells, larger microvessels and inflammatory cells. As described above, procedures for staining murine Factor VIII and murine CD34 are being developed and refined for our tissues in our laboratory. All tissues will subsequently be stained for endothelial cells to quantitate microvessel density with whichever of these two markers works better in our laboratory.

We have recently obtained a monoclonal antibody rat anti-mouse CD34 stain (Research Diagnostics, Inc., Flanders, NJ) but this has not worked in the formalin-fixed tissues, even with trypsin digestion for target retrieval. The supplier of this rat anti-murine CD34 stain recommends using a Tris-zinc fixative for the tissues (Beckstead, 1994). We have fixed some tissue samples with the Tris-zinc buffer and are presently testing this approach. NEN Life Science Products, Inc. (Boston, MA), the producer of the biotinyl tyramide reaction kit (product #NEL700), also recommends that detergents (such as Tween 80) not be used in buffers because the detergents may alter cell surface antigens such as Factor VIII and CD34. In future experiments, the biotinyl tyramide reaction kit will be used without Tween 80 in the wash buffer to stain for mouse CD34 on the endothelial cells.

Experiment #5: Presence of Steroid Receptors in Prostate Carcinoma Cell Lines.

The characteristics of the three most commonly used prostate carcinoma cell lines have recently been reviewed by Webber et al. (1997). There are still open questions about the role of steroid hormones on prostate carcinoma cells. In this experiment, we used RT-PCR to determine the presence of the glucocortical receptor, progesterone receptor and estrogen receptor α and β (ER α and ER β). (see Table 3).

Table 3. Identification of Steroid Receptors in Three Prostate Carcinoma Cell Lines. The PC3 tumor identifies PC3 cells grown in vivo in the nude mouse model.

Cell Line	ER α	ER β	Progesterone R	Glucocorticoid R
PC3	+	++	-	++
PC3 tumor	+	+	-	++
LNCaP	-	++	+	-
DU145	-	-	-	+

The PC3 cells had both ER α and ER β and glucocorticoid receptors. On the other hand, LNCaP cells, which are dependent on testosterone for growth, had no ER α or glucocorticoid receptor. However, it contained significant ER β and progesterone receptors. No steroid receptors were identified in DU145 cells except for a small amount of glucocorticoid receptor. These results suggest that the responses of the prostate carcinoma cell lines to the various steroid hormones are expected to be very different.

Experiment #6: Expression of Low Affinity Nerve Growth Factor (NGF) in Prostate Carcinoma Cells.

In the human prostate, a low affinity (p75) nerve growth factor (NGF) receptor is present in the epithelia while a NGF-like protein is present in the stroma. This NGF-like protein has been shown to participate in paracrine-mediated growth of a human tumor epithelial cell line (TSU-pr1) in vitro. Pflug et al. (1992) suggested that NGF receptor is differentially expressed in benign and malignant prostate tissue. We performed Western blot analysis using a primary antibody provided by Dr. Kathlyn Albers for the NGF receptor in subcellular fractions of prostate carcinoma cell lines and a HPV-transformed human prostate cell line (PZ-HPV-7 from ATCC). The NGF receptor was identified in the PC3 and LNCaP cell lines but not in DU145. In addition, PZ-HPV-7, a transformed prostate cell line, did not show NGF receptor protein.

Experiment #7. Metalloproteinase (MMP) Activity in Prostate Carcinoma Cell Lines.

Several reports have shown a correlation between MMP activity and metastasis in prostate carcinoma. MMP activity was measured in media of prostate carcinoma cell lines in vitro and in prostate tumors from the mouse xenograft using a polyacrylamide gel electrophoresis zymograph method described by Jung et al. (1998).

PC3 and DU145 cell cultures showed large amounts of MMP-9 activity but low MMP-2 activity in the media. On the other hand, no MMP activity was identified in culture media from LNCaP cells. MMP-2 activity was enhanced by addition of EGF (40 μ g/L) in DU145 but not in PC3 or LNCaP. When PC3 tumor cells obtained from the nude mouse xenograft were isolated and placed back in culture, the MMP activity was markedly different from the original PC3 cell line.

- Pro MMP-9 activity was not present in the xenograft cells.
- MMP-2 activity was sharply increased to similar activity as MMP-9 in tumor xenograft cells.
- NF681 and NF050 were potent inhibitors of MMP-2 and MMP-9 activity in PC3 cells.
- Suramin had no effect on MMP activity.

- Incubation of PC3-conditioned media with NF681 or NF050 did not affect MMP activity.

These results suggest that MMP activity may be an important factor in prostate carcinoma cells. This activity is inhibited by the phosphonic acid diphenylureas (NF681 and NF050) but not by suramin. Furthermore, the phosphonic acid diphenylureas do not directly inhibit MMP activity, suggesting that they may function by inhibition of the synthesis or secretion of MMPs. This is a very important and novel activity that has not been previously reported.

Experiment #8: Detection of Tissue Inhibitors of Metalloproteinases (TIMPs).

The presence of tissue inhibitors of metalloproteinases (TIMPs) in PC3 culture media was investigated using the reverse zymograph procedure. TIMP1 and TIMP3 were used as standards. No detectable TIMP activity was found in the media for any prostate cell lines (PC3, LNCaP or DU145) but was present in the prostate carcinoma cells. This TIMP activity was not altered by treatment with the phosphonic acid diphenylureas.

(7) Key Research Accomplishments

1. Demonstrated that the phosphonic acid diphenylureas are potent inhibitors of metalloproteinase activity in prostate carcinoma cells in vitro. The results suggest that this inhibition is not a direct effect on the MMP, but may be inhibition of MMP synthesis or secretion. This is the first compound reported to show this activity.
2. We have not been able to demonstrate inhibition of PC3 growth in the nude mouse xenograft. This may be because of solubility and drug delivery problems to the tumor site. We are developing new experiments to test these questions.

(8) Reportable Outcomes:

1. Abstracts and Presentations:

Gagliardi, A.R.T., P. Nickel and D.C. Collins. Inhibition of Prostate Cancer Cell Growth by Diphenylureas, A Unique Group of Antiangiogenic Compounds. Proceedings of the 17th UICC International Cancer Congress, pp. 1109-1113, 1998.

Gagliardi, A.R., M.C. Bittencourt, R.K. Munn, P. Nickel and D.C. Collins. Inhibition of Renal Carcinoma Cell Growth by Diphenylureas, a Unique Group of Antiangiogenic Compounds. Proceedings of the 17th UICC International Cancer Congress, pp.1137-1140, 1998.

Gagliardi, A.R.T., P. Nickel and D.C. Collins. Inhibition of Prostate Cancer Cell Growth by Diphenylureas, A Unique Group of Antiangiogenic Compounds. American Association for Cancer Research, "Novel Approaches to Organ Site-Specific Therapies" Minisymposium, 1998.

Gagliardi, A.R., R.K. Munn, P. Nickel and D.C. Collins. Inhibition of Prostate Cancer Cell Growth by a Unique Group of Antiangiogenic Compounds. Fourth International Symposium on Predictive Oncology and Therapy, Nice, France, 1998.

Collins, D.C., A.R.T. Gagliardi and P. Nickel. Inhibition of Prostate Cancer Growth, Metalloproteinase (MMP) Activity and Urokinase-Plasminogen Activator (uPA) by Phosphonic Acid Diphenylureas. American Association for Cancer Research Symposium "New Research Approaches in the Prevention and Cure of Prostate Cancer", abstract, 1998.

Gagliardi, A.R., M. Bittencourt, R. Munn, P. Nickel and D.C. Collins. Inhibition of Renal Cell Carcinoma Growth In Vitro by Phosphonated Diphenylureas. American Association for Cancer Research, abstract, 1999.

Gagliardi, A., D.C. Collins and P. Nickel. Phosphonic Acid Diphenylureas, A Unique Group of Potent Antiangiogenic Compounds. Beatson International Cancer Conference, "Invasion and Metastasis", abstract, 1999.

2. Patents:

Patent Application. Phosphonic Acid Diphenylureas . Methods for Synthesis, Antiangiogenic and Antitumorigenic Activity. Submitted with Antonio R.T. Gagliardi, M.D., Ph.D. and Peter Nickel, Ph.D.

(9) Conclusions:

In Year 1 of this grant, we are establishing procedures for determining the ability of the phosphonic acid diphenylureas to inhibit PC3 prostate carcinoma cells in the nude mouse xenograft. The initial experiments have not confirmed that the phosphonic acid diphenylureas inhibit prostate cancer in the nude mouse xenograft. Additional experiments are necessary to determine the reason for the differences in results in the in vitro and in vivo experiments. We postulate that the i.p. injection of the treatments into the peritoneal cavity does not deliver the phosphonic acid diphenylureas to the tumor.

In other experiments, the phosphonic acid diphenylureas have been shown to be potent inhibitors of metalloproteinase (MMP) activity in prostate carcinoma cells. We postulate that these compounds inhibit MMP secretion or synthesis. Additional experiments are proposed to test this hypothesis.

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Inhibition of prostate cancer cell growth by diphenylureas, a unique group of antiangiogenic compounds

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SUMMARY

We have synthesized and characterized a unique group of phosphonated diphenylureas that are potent inhibitors of angiogenesis. The three most active phosphonated diphenylureas were equal to or more potent than suramin or its sulfonated analogues as inhibitors of growth by human prostate cancer cell lines (LNCap, PC3, and DU145). They were also potent inhibitors of the metalloproteinases, gelatinase A (MMP-2) and gelatinase B (MMP-9) in human prostate cancer cell lines. These results suggest that these unique compounds may be effective therapeutic agents for prostate cancer.

INTRODUCTION

Our laboratory first showed that suramin is a potent inhibitor of angiogenesis (Gagliardi et al., 1992). Suramin is currently undergoing phase II and III trials in prostate cancer and various solid tumors. Since the initial reports of suramin activity in metastatic hormone refractory prostate cancer, studies have confirmed that suramin has independent antitumor activity in hormone refractory prostate cancer (Myers et al. 1992). A major

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vitro. We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some prostate cancer cell lines in vitro. The IC₅₀ values for suramin and the most active diphenylureas on the classical prostate cancer cell lines, LNCaP (androgen dependent), PC3 and DU145, are shown in **Table 1**. The three most active diphenylureas (NF681, NF162 and NF050) showed IC₅₀ values equal to or greater than suramin and its sulfonated analogues in LNCaP, PC3 and DU145 human prostate cancer cells.

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Table 1: The IC₅₀ values for growth of human prostate cancer cell lines treated with suramin and selected diphenylureas as determined with the MTT assay by incubating with various concentrations of suramin and diphenylureas for five days.

Cell Lines	PC3	LNCaP	DU145
Compound (μM)			
Suramin	125	300	>500
NF681	62	225	250
NF167	150	500	500
NF162	50	320	500
NF069	62	350	500
NF050	50	350	500

Inhibition of matrix metalloproteinases (MMP) by diphenylureas.

Polyacrilamide gel electrophoresis zymography of serum free media of cultures from PC3 prostate cancer cells contained significant amounts of 72-KD gelatinase A (MMP-2) activity. DU145 prostate cancer cells secreted 92-KD gelatinase B (MMP-9). The secretion of these metalloproteinases was enhanced in both cell types by the addition of Epidermal Growth Factor (EGF). NF681, NF167 and NF050 were potent inhibitors of gelatinase A in the PC3 cells and gelatinase B in DU145 cells.

DISCUSSION

We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some tumor cell lines in vitro. The three most active diphenylureas (NF681, NF162 and NF050) showed IC₅₀ values equal to or greater than suramin and its sulfonated analogues in LNCaP, PC3 and DU145 human prostate cancer cells. These results clearly indicate that the diphenylureas are potent inhibitors of the growth of prostate cancer cell lines. Several recent reports have shown a correlation between MMP expression and tumor invasiveness in prostate cancer. Pajouh et al. (1991) found that MMP-7 was increased in malignant compared to benign prostatic tissue but absent in stroma. Boag and Young (1993) found increased levels of gelatinase A (MMP-2) in malignant prostate and metastatic tissue. Stearns and Wang (1993) analyzed prostate cancer tissue extracts for gelatinase A (MMP-2) and found that the enzyme is selectively over-expressed by malignant pre-invasive epithe-

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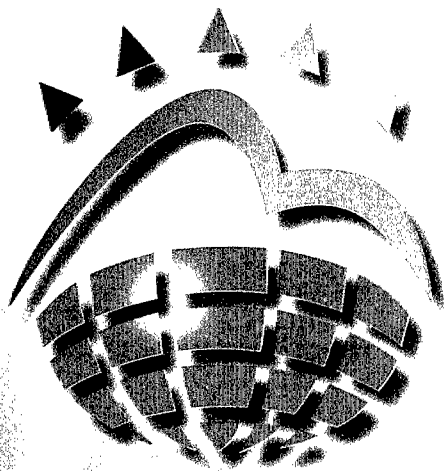
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Inhibition of renal carcinoma cell growth by diphenylureas, a unique group of antiangiogenic compounds

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SUMMARY

We have synthesized and characterized a unique group of phosphonated diphenylureas that are potent inhibitors of angiogenesis. We found that four of the most active phosphonated diphenylureas were equal to or more potent than suramin as inhibitors of growth by human renal cancer cell lines (A-498, 786-0, Caki-1 and Caki-2). They were also potent inhibitors of the metalloproteinase, gelatinase A (MMP-2), in human 786-0 renal cancer cells. These results suggest that these unique compounds may be effective therapeutic agents for prostate cancer.

INTRODUCTION

We have synthesized and characterized a unique group of phosphonated diphenylurea compounds with molecular weights about half that of suramin. The diphenylureas are up to 30 times more active than suramin as inhibitors of

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angiogenesis in the chick chorioallantoic membrane (CAM) assay. Furthermore, nonspecific binding of these compounds to serum proteins is lower and the half lives shorter than for suramin or its sulfonated analogues. In this study, the effect of selected phosphonated diphenylureas was determined on the growth of human renal cancer cell lines (A-498, 786-0, Caki-1, Caki-2) in vitro. In addition, their effect on matrix metalloproteinase activity in the human renal cancer cells was determined.

MATERIALS AND METHODS

Cell Culture: Human renal cancer cell lines, A-498, 786-0, Caki-1 and Caki-2, were obtained from ATCC (Rockville, MD). Cells were cultured in a complete growth media. A-498 was cultured in DMEM containing 10% heat inactivated FCS, 1% NEAA, 1mM L-glutamine, 100 units/ml streptomycin. 786-0 cells were cultured in RPMI medium supplemented with 10%FCS, 1 mM sodium pyruvate, 1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Caki-1 and Caki-2 cells were cultured in McCoy's medium containing 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin.

Effect on Growth In Vitro: Renal cancer cells were harvested by trypsinization, resuspended in fresh medium and 100 µl (3000-5000 cells/well) of the cell suspension were plated in 96 well flat bottom microtiter plates (Corning, NY) and incubated in a humidified atmosphere with 5% CO₂ at 37° C. Suramin or the diphenylureas (0-500 µM) were added to the cell culture after 24 hr and incubated for 5 days. Cell growth curves were used to calculate the 50% inhibitory concentration (IC₅₀) for suramin and the diphenylureas. The MTT assay described by Carmichael et al. (1987) was used to determine growth rate. Because suramin in the medium reduced formazan production in the MTT assay, wells were washed twice with PBS before MTT was added for 3 hr.

Metalloproteinase activity: SDS-polyacrilamide gel electrophoresis (PAGE) using 8% gels containing 0.1% gelatin was used to determine metalloproteinase activity in renal cancer cells in vitro as described by Lim et al. (1996).

RESULTS

Effects of the diphenylureas on the growth of renal cancer cell lines in vitro.

We have compared the inhibitory activity of the diphenylureas and suramin on proliferation of some renal cancer cell lines in vitro. The IC₅₀ values for suramin and the most active diphenylureas on the classical renal cancer cell lines, A-498, 786-0, Caki-1 and Caki-2, are shown in Table 1. The two most active diphenylureas (NF681 and NF162) showed IC₅₀ values equal to or greater than suramin and its sulfonated analogues in the renal cancer cell lines. NF681 showed the greatest inhibitory activity with IC₅₀ values of 15 and 13 µM in A-498 and 786-0 renal cancer cells, respectively. However, the values for Caki-1 and Caki-2 cells were >500 µM, indicating relatively little inhibitory activity. The IC₅₀ values for NF162 ranged from 138-226 µM in the four renal cancer cell lines. These results compared favorably with the values for suramin and seemed to inhibit all of the renal cancer cells tested. These results clearly indicate that the diphenylureas are potential therapeutic agents for inhibiting the growth of human renal cancer in vitro.

Table 1: The IC₅₀ values for growth of human renal cancer cell lines treated with suramin and selected diphenylureas as determined with the MTT assay by incubating with various concentrations of suramin and diphenylureas for five days.

Cell Lines	A-498	786-0	Caki-1	Caki-2
Compound (μM)				
Suramin	>500	>254	56	>500
NF050	>500	>500	31	>500
NF069	166	>500	370	>500
NF162	138	200	100	226
NF681	15	13	>500	>500

Inhibition of matrix metalloproteinases (MMP) by diphenylureas.

Polyacrilamide gel electrophoresis zymography of serum free media conditioned by cultures of 786-0 cells contained 72-KD gelatinase A activity (MMP-2) but no 92-KD gelatinase B activity (MMP-9). The gelatinase A activity was enhanced by Epidermal Growth Factor (EGF) (40 ng/ml). Treatment with NF681 and NF050 (35, 70, 140 μM) for 24 hr induced a significant inhibition of gelatinase A activity and abolished the stimulation of EGF. This inhibition was not seen when suramin was added. The A498 cell line did not express detectable metalloproteinase activity in vitro.

DISCUSSION

Renal cell carcinoma remains one of the major challenges for oncologists (Young, 1997). Over the past years, progress has been made in understanding the molecular biology of this disease as new insights in prognostic factors for outcome following nephrectomy. The results of systemic therapy for metastatic renal cell carcinoma are disappointing and currently available chemotherapeutic agents have not been effective. Even promising new anti-metabolite compounds resulted in responses of less than 10% of the treated patients. The most important characteristics of renal cell carcinoma include hypervascularity, high frequency of metastasis and poor prognosis. The role of angiogenesis in growth of renal cancer cells and metastasis has been shown in clinical studies (Yoshino et al., 1995). Microvessel count is a significant predictor of survival and the density of microvessels correlates with metastasis.

Our results show that diphenylureas are potent inhibitors of renal cell carcinoma growth in vitro. These compounds are effective inhibitors of angiogenesis in the chick chorioallantoic membrane (CAM) assay and inhibit human microvascular endothelial cell growth and tube/cord formation in Matrigel.

The diphenylureas also inhibit gelatinase activity in conditioned media from renal cancer cells. Recent studies provided evidence that progelatinase A mRNA expression in cell lines derived from tumors in patients with renal cancer correlates inversely with survival (Walther et al., 1997). Gelatinase A activity correlates with tumor invasiveness in vivo and synthetic inhibitors of gelatinase activity prolonged survival and decreased the number of metastasis. This

suggests that the inhibition of metalloproteinase activity may be a suitable target for therapeutic intervention in human renal cancer.

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“New Research Approaches in the Prevention and Cure of Prostate Cancer”

Inhibition of Prostate Cancer Cell Growth, Metalloproteinase (MMP) Activity and Urokinase-Plasminogen Activator (uPA) by Phosphonic Acid Diphenylureas. Delwood C. Collins, Antonio R.T. Gagliardi, and Peter Nickel. VA Medical Center and University of Kentucky College of Medicine, Lexington, KY and University of Bonn, Bonn, Germany.

We have synthesized and characterized a unique group of phosphonic acid diphenylurea compounds. These compounds have molecular weights half that of suramin, are less bound to plasma proteins and have shorter half-lives than suramin. When suramin or the phosphonic acid diphenylureas was added to cultures (0-400 μ M) of LNCaP, PC3, or DU145 prostate cancer cells, cell proliferation was significantly inhibited for all three cell lines. The three most active phosphonic acid diphenylureas (NF681, NF050, NF162) showed 50% inhibitory concentrations (IC_{50}) similar to or less than suramin for all three cell lines.

MMPs are important in the regulation of prostate tumor progression. The 72-kDa (MMP-2) and the 92-kDa (MMP-9) have been reported to play a crucial role in the degradation of basement membrane type-IV collagen, resulting in increased cancer metastasis. In these experiments, the effect of the phosphonic acid diphenylureas (50-400 μ M) and suramin on MMP-2 and MMP-9 activities was evaluated by SDS-polyacrylamide gel electrophoresis zymography (0.1% gelatin). Phosphonic acid diphenylurea treatment for 24 hr markedly decreased MMP-9 activity in PC3 cells and both MMP-2 and MMP-9 activities in DU145 cells. Treatment with suramin showed no effect.

Plasminogen activators, a class of serine proteases, are important in local tumor growth, invasion and metastasis. Increased uPA in prostate tumors displaying invasive growth supports the hypothesis that uPA plays a key role in tumor invasion and metastasis. The effect of phosphonic acid diphenylureas on uPA activity was also evaluated by SDS-polyacrylamide gel electrophoresis zymography (0.1% casein). Treatment with the phosphonic acid diphenylureas for 24 hr inhibited uPA activity significantly in PC3 cells in a dose-related manner.

These results suggest that the phosphonic acid diphenylureas have therapeutic potential for prostate cancer by inhibiting cell growth, metalloproteinase and uPA activities.
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#4386 **Inhibition of prostate cancer cell growth by diphenylureas, a unique group of antiangiogenic compounds.** Gagliardi, A.R.T., Nickel, P.; and Collins, D.C. *VA Medical Center and the University of Kentucky College of Medicine, Lexington, KY 40536 and the University of Bonn, Bonn, Germany.*

We have synthesized and characterized a group of diphenylurea compounds that are potent inhibitors of angiogenesis. The diphenylureas have molecular weights about half that of suramin, are less bound to plasma proteins and have a shorter half-life than suramin. Toxicity studies in mice showed that they are less toxic than suramin. The diphenylureas are up to 30 times more potent than suramin as inhibitors of angiogenesis in the chick chorioallantoic membrane assay and 100 times more active as inhibitors of human microvascular endothelial cell growth, migration and in vitro (Matrigel) tube formation. The MTT assay was used to examine the effects of selected diphenylureas on LNCaP, PC3 and DU145 cell proliferation. The three most active diphenylureas had 50% inhibitory concentrations (IC_{50}) equal to or less than suramin and its sulfonated analogues. Furthermore, the diphenylureas are potent inhibitors of gelatinase B (MMP-9) activity in PC3 cells and gelatinase A (MMP-2) and B activity in DU145 cells in culture. Our results suggest that the diphenylureas have therapeutic potential for prostate cancer by inhibiting prostate cancer cell growth, tumor and matrix metalloproteinase activity and tumor angiogenesis. (Supported by the Department of Veterans Affairs and the American Cancer Society.)

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Inhibition of prostate cancer cell growth by a unique group of antiangiogenic compounds.

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AIM: We have synthesized and characterized a group of diphenylurea compounds that are potent inhibitors of angiogenesis. The diphenylureas have molecular weights about half that of suramin, are less bound to plasma proteins and have a shorter half-life than suramin. Toxicity studies in mice showed that they are less toxic than suramin. The diphenylureas are up to 30 times more potent than suramin as inhibitors of angiogenesis in the chick chorioallantoic membrane assay and 100 times more active as inhibitors of human microvascular endothelial cell growth, migration and in vitro (Matrigel) tube formation.

METHODS: The MTT assay was used to examine the effects of selected diphenylureas on LNCaP, PC3 and DU145 cell proliferation. Metalloproteinase activity was determined by polyacrylamide gel electrophoresis zymography.

RESULTS: The three most active diphenylureas had 50% inhibitory concentrations (IC₅₀) equal to or less than suramin and its sulfonated analogues. Furthermore, the diphenylureas are potent inhibitors of gelatinase B (MMP-9) activity in PC3 cells and gelatinase A (MMP-2) and B activity in DU145 cells in culture.

CONCLUSIONS: Our results suggest that the diphenylureas have therapeutic potential for prostate cancer by inhibiting prostate cancer cell growth, tumor and matrix metalloproteinase activity and tumor angiogenesis. (Supported by the Department of Veterans Affairs and the American Cancer Society.)

KEY WORDS: diphenylureas, antiangiogenesis, metalloproteinase, prostate cancer